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Cytotoxicity of red fluorescent protein DsRed is associated with the suppression of Bcl-xL translation

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ABSTRACT

Red fluorescent protein (RFP) DsRed and its variants are widely applied in live-cell imaging experiments. However, a major factor that restricts the application of DsRed is its cytotoxicity. Here, we report that DsRed and its variant DsRed-Express2 inhibit the expression of B-cell lymphoma-extra large (Bcl-xL) in HeLa cells by translational regulation. Over-expression of Bcl-xL can reduce the cytotoxicity of DsRed. Meanwhile, Turbo RFP, a mutant RFP from *Entacmaea quadricolor*, does not affect Bcl-xL expression, suggesting that cytotoxic mechanisms of RFP from different species may be varied. Our results reveal a possible mechanism for the cytotoxicity of DsRed, providing a potential strategy to improve the application of DsRed and its variants.

1. Introduction

DsRed is a red fluorescent protein (RFP) from coral *Discosoma* sp. [1], with the excitation and emission maxima at 558 and 583 nm, respectively [2]. DsRed is homologous to green fluorescent protein (GFP), which forms an 11-strand β barrel and a chromophore embedded inside the barrel [3]. It has a much higher extinction coefficient and fluorescent quantum yield compared to GFP, and it quite resists to photo bleaching with a wider pH tolerance range (4.5–12). These advantages attracted tremendous interests for applications in live-cell imaging [2,4].

Despite the great potential in application, DsRed has several drawbacks. First, the maturation of DsRed is extremely slow, which may take days at room temperature. Secondly, DsRed is prone to oligomerization and aggregation [5]. Finally, the cytotoxicity of wild type DsRed and its variants severely limits its application. Although several improved variants such as DsRed-Monomer [5], DsRed.T4 [6], and DsRed2 [7] have been developed by site-direct mutagenesis, cells expressing high levels of DsRed or its variants still show growth defects and/or instability [8–10]. This problem is much more prominent in DsRed compared to GFP and other green fluorescent variants. Although it was speculated that the cytotoxicity was caused by the aggregation of DsRed proteins [10], the molecular mechanism of the DsRed-mediated cytotoxicity remains to be elucidated [11].

B-cell lymphoma-extra large (Bcl-xL) and B-cell lymphoma 2 (Bcl-2) are members of Bcl-2 protein family [12]. They are highly similar both in protein sequence and structure. Both of them are antiapoptotic proteins, which help cells to be more resistant to apoptosis [13,14]. The expression of Bcl-xL and Bcl-2 is up-regulated in many types of cancer cells [15]. Inhibitors of Bcl-xL and Bcl-2 can induce apoptosis or autophagic cell death in cancer cells [16,17]. Besides, Bcl-xL and Bcl-2 are normally localized to mitochondrial membranes because the C-terminal of proteins contains a mitochondrial signal, targeting them to the mitochondria [15].

Here we report that DsRed and its variant DsRed-Express2 inhibit the expression of Bcl-xL protein in HeLa cells. Meanwhile,

Abbreviations: RFP, red fluorescent protein; GFPgreen fluorescent protein; DMEM, Dulbecco' s modified eagle medium; DMI, digital microscope inverted; DFC, digital firewire camera; CCD,charge-coupled device; Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma-extra large

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over-expression of Bcl-xL prevents the cytotoxicity of DsRed. Our results may provide a potential strategy to alleviate cytotoxic problem of DsRed and its variants.

2. Materials and methods

2.1. Plasmids construction

Vectors of Wassabi GFP and pDsRedN1 were purchased from Allele Biotech (USA) and Clontech (USA), respectively. Turbo RFP plasmid was obtained from Origene (USA). DsRed-Express2 was provided by Dr. Benjamin S. Glick. Synthetic oligonucleotide primers were listed in Supplementary Table 1. Bcl-xL and Bcl-2 cDNA were kept in our lab. Bcl-xL fragment with restrictions enzyme sites XhoI and EcoRI was generated by PCR with primers ZJ01n and ZJ02c. Bcl-2 fragment with restrictions enzyme sites XhoI and EcoRI was generated by PCR with primers ZJ03n and ZJ04c. Both Bcl-xL and Bcl-2 fragments were ligated into the vector of WasabiC GFP. GFP-(∗)-Bcl-xL plasmid (∗ indicated a stop code) was generated from GFP-Bcl-xL plasmid by site-directed mutagenesis kit (Beijing SBS Genetech, China) with the primers ZJ05n and ZJ06c.

2.2. Cell culture and transfection

HeLa cells were maintained in a humidified incubator at 37 °C with 5% CO_2 and grown in Dulbecco's modified eagle medium containing 10% fetal calf serum (Hyclone, USA). Cells were plated into 24-well tissue culture plates. After the density of cells reached 70%, cells were transiently transfected with plasmids as described using Lipofectamine 2000 (Invitrogen, USA).

2.3. Fluorescent image

Fluorescent cells were observed using a Leica Digital Microscope Inverted 6000B inverted fluorescence microscope. The images were captured by a Leica digital firewire camera 420 charge-coupled device under a 40× objective and recorded on a PC using Leica Application Suite (v2.5.0 R1).

2.4. Western blotting

Cells were transfected with plasmids as indicated in the Section 3. After 36 h, cells were harvested and lysed by cell lysis solution (Beyotime, China) for Western blotting analysis. The antibodies for assays were anti-Bcl-xL mAb (Invitrogen, USA) diluted 1:200, anti-His mAb (Santa Crutz, USA) diluted 1:1000, anti-β-actin mAb (Santa Crutz, USA) diluted 1:1000, and goat-anti-mouse IgG (Santa Crutz, USA) diluted 1:4000.

2.5. Transcription regulation assay of Bcl-xL in HeLa cells and RT-PCR

Total RNA in cells co-transfected with plasmids encoding GFP-Bcl-xL and DsRed or empty vector, was extracted by TRNzol (TianGen, China). RT-PCR was used to amplify a fragment of cDNA. The primers which used for amplifications of indicated fragments were listed in Supplementary Table 2. ZJ07n and ZJ08c were for generating exogenous Bcl-xL fragments which is 492 bp from nt 482 of GFP to nt 296 of Bcl-xL. ZJ09n and ZJ10c were for generating endogenous Bcl-xL fragments which is 202 bp from nt 400 to nt 602. ZJ11n and ZJ12c were for generating GAPDH fragments which is 372 bp.

2.6. Apoptosis assay

Apoptosis was detected by Hoechst 33342 staining kit (Beyotime, China). The condensed chromatin of apoptotic cells were stained brightly by Hoechst 33342, while the normal chromatin of live cells were stained more weakly, which makes it possible to distinguish normal and apoptotic cells under fluorescence microscopy.

2.7. Fluorescence intensity assay

HeLa cells were transiently co-transfected by various construct plasmids accordingly. 20 000 cells were sorted by flow cytometry (FacsAir flow cytometer, BD Biosciences) under identical condition (488 nm laser for GFP and 543 nm laser for RFPs). Data were processed using Flow cytometry software Summit V4.0.

To measure the growth of cells expressing fluorescent proteins, DsRed, DsRed-Express2 and Turbo RFP plasmids were co-transfected with pcDNA4.0 and pcDNA4.0-Bcl-xL plasmids respectively. Cells of four wells in a 24-wells plate were harvested at specific time from 12 to 84 h after transiently transfection. Viable fluorescence cells were counted and analyzed from 20 000 cells sorted by flow cytometry. Data were processed using Flow cytometry software Summit V4.0.

3. Results and discussion

3.1. DsRed and its variant DsRed-Express2 down-regulates Bcl-xL protein in HeLa cells

When we co-transfected plasmids encoding DsRed and GFP-Bcl-xL into HeLa cells, we accidentally observed that green fluorescence intensity of cells expressing both DsRed and GFP-Bcl-xL (Fig. 1b2) was much weaker than that of cells expressing GFP-Bcl-xL only (Fig. 1a2). However, there was no obvious difference in green fluorescence intensity between cells expressing both DsRed and GFP (Fig. 1b1) and cells expressing GFP only (Fig. 1a1). Thus, it seems that the green fluorescence could be suppressed by DsRed when GFP is fused to Bcl-xL. As Bcl-2 is a homologous protein of Bcl-xL, we also tried to co-transfect plasmids encoding DsRed and GFP-Bcl-2 into HeLa cells. No obvious difference in green fluorescence intensity was observed between cells expressing both DsRed and GFP-Bcl-2 (Fig. 1b3) and cells expressing GFP-Bcl-2 only (Fig. 1a3). Thus, it seems that the effect of DsRed is specific for Bcl-xL.

Since DsRed-Express2 was reported to be an improved variant of DsRed [10], we co-transfected plasmids encoding GFP-Bcl-xL and DsRed-Express2. The green fluorescence intensity of cells expressing both DsRed-Express2 and GFP-Bcl-xL (Fig. 1f2) was also much weaker than that of cells expressing GFP-Bcl-xL only (Fig. 1a2). And there was no decline of green fluorescence intensity in cells expressing DsRed-Express2 and GFP (Fig. 1f1) or in cells expressing GFP-Bcl-2 and DsRed-Express2 (Fig. 1f3).

To further ascertain the green fluorescence intensity of GFP-Bcl-xL was decreased by DsRed and its variant DsRed-Express2, we examined green fluorescence of cells by flow cytometry. The average green fluorescence intensity was obviously decreased by overexpression of DsRed and DsRed-Express2 (Fig. 2A). The normalized green fluorescence intensity was decreased to 22.45 and 30.46, respectively (Fig. 2B).

We then carried out western blotting to analyze the protein expression level of GFP-Bcl-xL. The results showed that the amount of GFP-Bcl-xL protein is significantly lower in cells expressing GFP-Bcl-xL and DsRed than that in cells expressing GFP-Bcl-xL only (Fig. 3). Similar results were also obtained in cells expressing GFP-Bcl-xL and DsRed-Express2 (Fig. 3). Further, we found that the endogenous Bcl-xL protein levels were also lowered in HeLa cells co-transfected with plasmids encoding DsRed or DsRed-Express2 with GFP-Bcl-xL (Fig. 3). Therefore, the over-expression

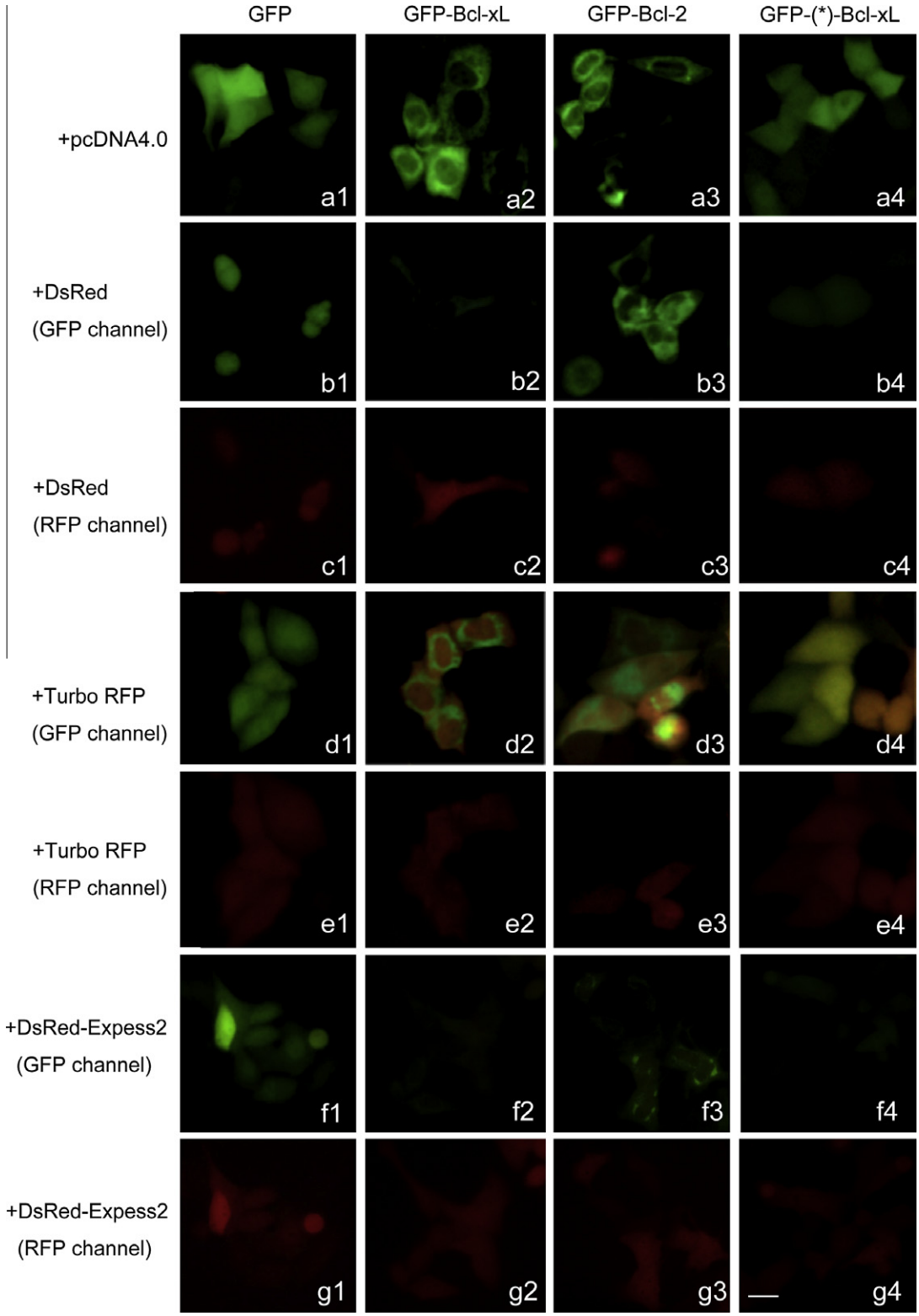

Fig. 1. Fluorescent image results showing that inhibition of green fluorescence of GFP-Bcl-xL or GFP(∗)-Bcl-xL by DsRed or DsRed-Express2 in HeLa cells. (a1–g4) Representative fluorescence images of HeLa cells co-transfected with plasmids expressing different combination of proteins as indicated in the panel. Over-expression of DsRed or DsRed-Express2 can decrease the green fluorescence intensity of GFP-Bcl-xL (b2 and f2). Meanwhile, over-expression of DsRed or DsRed-Express2 does not decrease green fluorescence intensity of GFP (b1 and f1) or GFP-Bcl-2 (b3 and f3). Over-expression of Turbo RFP does not decrease green fluorescence intensity of GFP (d1), GFP-Bcl-xL (d2), and GFP-Bcl-2 (d3). The green fluorescence intensity in cells expressing both DsRed/DsRed-Express2 and GFP(∗)-Bcl-xL (∗ stop codon) was also weaker (b4 and f4) than that in cells expressing GFP(∗)-Bcl-xL alone (a4). However, there was no obvious difference in green fluorescence intensity between cells expressing Turbo RFP and GFP (d1) and cells expressing Turbo RFP and GFP(∗)-Bcl-xL (d4). Exposure time for green fluorescent images was 800 ms. Exposure time for red fluorescent images of Turbo RFP was 800 ms. Exposure time for red fluorescent images of DsRed was 2 s due to weaker fluorescence. The bar represents 20 μm.

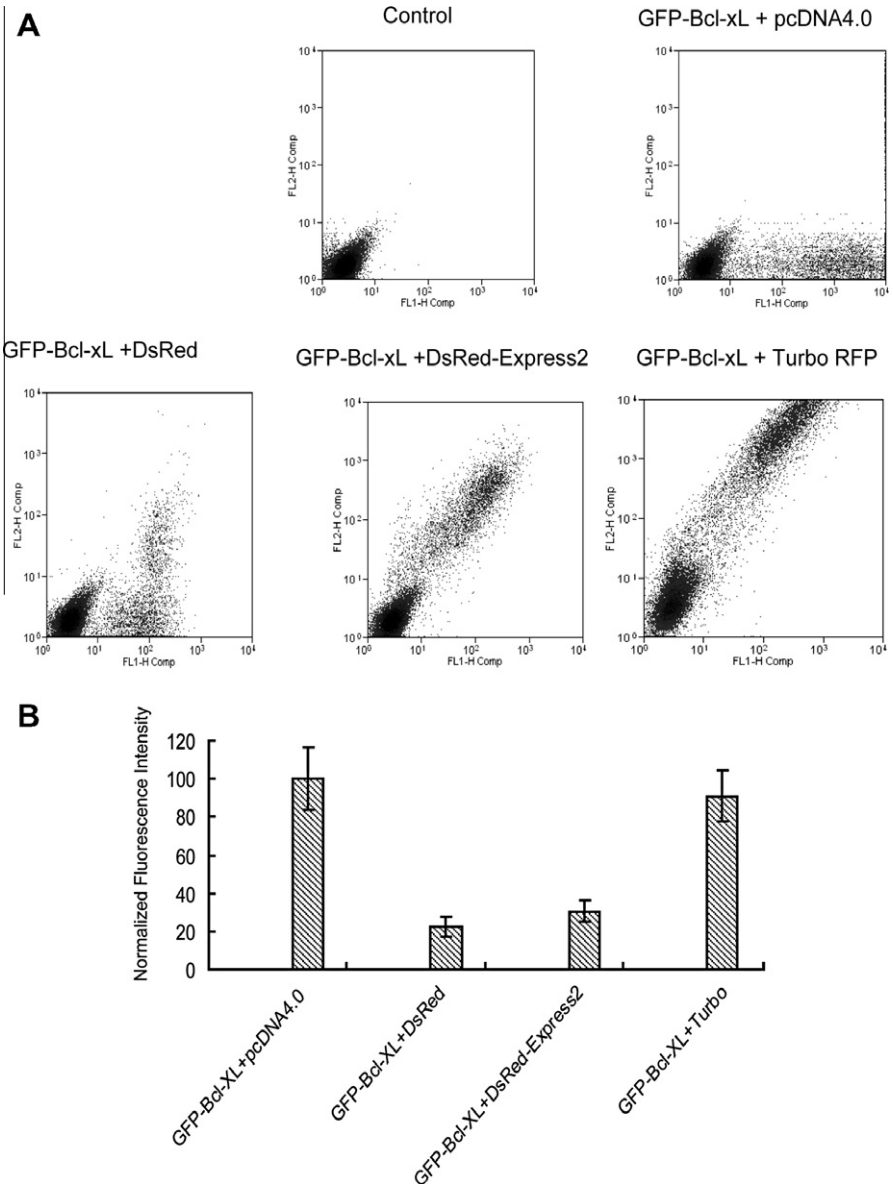

Fig. 2. Flow cytometry results showing that inhibition of green fluorescent intensities of GFP-Bcl-xL by DsRed or DsRed-Express2 in HeLa cells. A. GFP-Bcl-xL plasmids were transiently co-transfected with plasmids as indicated in HeLa cells. Green fluorescent cells were counted by flow cytometry at 48 h. The data were analyzed by the software summit 4.0. (B) Normalized fluorescence intensity of cells transfected with plasmids as indicated in HeLa cells. The strongest green fluorescence intensity obtained for cells expressing GFP-Bcl-xL alone was defined as 100 units.

of DsRed or DsRed-Express2 can result in lowered endogenous Bcl-xL and exogenous GFP-Bcl-xL protein levels, which explains the lowered green fluorescence intensity in HeLa cells.

3.2. DsRed represses the expression of Bcl-xL by translational regulation

To decrease the GFP-Bcl-xL protein level, DsRed could act to accelerate the protein degradation, or down-regulate either the protein or the mRNA production. To distinguish these possibilities, we constructed a plasmid encoding GFP(∗)-Bcl-xL, in which a stop codon was inserted between GFP and Bcl-xL coding sequences so that only GFP protein could be produced even though the mRNA contained the coding sequence of Bcl-xL. Interestingly, when plasmids encoding DsRed and GFP(∗)-Bcl-xL were co-transfected into HeLa cells, the green fluorescence intensity was still weaker than that of cells expressing DsRed and GFP (Fig. 1b4 and b1). Similar results were also observed in cells expressing DsRed-Express2 and GFP(∗)-Bcl-xL (Fig. 1f4). Considering that DsRed or DsRed-Express2 does not affect GFP protein production when there is no Bcl-xL coding sequence (Fig. 1b1 and g1), these results suggest that DsRed or DsRed-Express2 represses expression of Bcl-xL by transcription or translational regulation.

We next investigated whether DsRed inhibited the transcription of Bcl-xL in HeLa cells. We extracted the total RNA of HeLa cells co-transfected with plasmids encoding GFP-Bcl-xL and DsRed or empty vector, and used RT-PCR to amplify a fragment of GFP-Bcl-xL cDNA. As shown in Fig. 4A, DsRed did not inhibit the transcription of GFP-Bcl-xL mRNA, as the band intensities of RT-PCR products are comparable. We then transfected plasmids encoding DsRed or empty vector into HeLa cells, and examined whether the transcription of endogenous Bcl-xL was affected by the over-expression of DsRed. Same as exogenous results, DsRed also did not inhibit the transcription of endogenous Bcl-xL (Fig. 4B). These

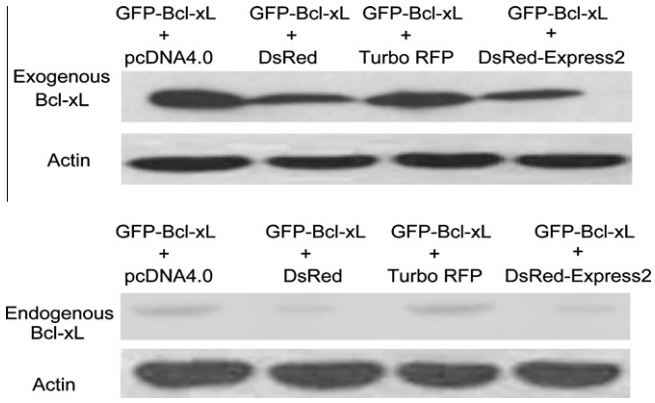

Fig. 3. Inhibition of Bcl-xL expression by DsRed or DsRed-Express2 in HeLa cells. Down-regulation of both exogenous and endogenous expression of Bcl-xL by the overexpression of DsRed and DsRed-Express2. HeLa cells co-transfected with plasmids expressing GFP-Bcl-xL together with DsRed, DsRed-Express2 or Turbo RFP. The expression of both exogenous (upper panel) and endogenous of Bcl-xL (lower panel) was determined by western blotting.

results suggest that DsRed could repress the expression of Bcl-xL by translational regulation.

3.3. Over expression of Bcl-xL reduces the cytotoxicity of DsRed and DsRed-Express2 in HeLa cells

We next evaluated the effect of Bcl-xL on DsRed-mediated cytotoxicity in HeLa cells. When cells were transfected with plasmids encoding DsRed alone, 51.7% of red fluorescent cells became shriveled and round after 48 h. However, when cells were transfected with plasmids encoding both DsRed and Bcl-xL, only 9% red fluorescent cells appeared to be shriveled and round after 48 h (Fig. 5A). As a control, only 6% of green fluorescent cells appeared to be shriveled. Since Bcl-xL is an important negative-regulator of apoptosis, we analyzed the effect of Bcl-xL on DsRed-elicited apoptosis by using Hochest 33342. The percentage of apoptotic cells was about 11.25% in cells which were transfected with plasmids encoding DsRed alone, while it was decreased to 3.4% in cells transfected with plasmids encoding both DsRed and GFP-Bcl-xL (Fig. 5B and C).

DsRed-Express2 was reported to be the best variant derivative of DsRed. The fluorescence maturation, expression, photostability and photoxicity were much more improved compared with DsRed [10]. When cells were transfected with plasmids encoding DsRed-Express2 alone, there are also 23.5% of red fluorescent cells became shriveled and round after 48 h. However, when cells were transfected with plasmids encoding both DsRed-Express2 and Bcl-xL, only 3.8% red fluorescent cells appeared to be shriveled and round after 48 h (Fig. 5A). Apoptosis analysis by Hochest 33342 showed the percentage of apoptotic cells was about 6.3% in cells which were transfected with plasmids encoding DsRed-Express2 alone, while it was decreased to 3.5% in cells transfected with plasmids encoding both DsRed-Express2 and Bcl-xL (Fig. 5B and C).

To further evaluate Bcl-xL on the inhibition of DsRed or DsRed-Express2-elicited cytotoxicity, percentage of fluorescent cells was counted by flow cytometry at the time from 12 to 84 h after transfections (Supplementary Fig. 1). Over-expression of Bcl-xL did not increase percentage of green fluorescent cells (Supplementary Fig. 1A). However, over-expression of Bcl-xL obviously increased percentage of red fluorescent cells expressing DsRed (Supplementary Fig. 1B). Besides, numbers of red fluorescent cells expressing DsRed-Express2 was also increased (Supplementary Fig. 1C).

Our results indicate that the cytotoxicity of DsRed and DsRed-Express2 is correlated with the down regulation of Bcl-xL, while overexpression of Bcl-xL can decrease the cytotoxicity of DsRed and DsRed-Express2 in HeLa cells.

3.4. Turbo RFP does not inhibit the expression of Bcl-xL protein

Turbo RFP and its mutant TagRFP are also widely applied in red fluorescent imaging. Turbo RFP is a dimeric RFPfrom *Entacmaea quadricolor*, and it is much brighter than DsRed [18]. We also investigated whether Turbo RFP inhibited the fluorescence of GFP-Bcl-xL or GFP-Bcl-2. The fluorescence image results showed that Turbo RFP did not inhibit the green fluorescence intensity of GFP-Bcl-2, GFP-Bcl-xL and GFP-(∗)-Bcl-xL in HeLa cells (Figs. 1d2–d4, and 2). Further, western blot results confirmed that Turbo RFP did not inhibit the expression of Bcl-xL protein in HeLa cells (Fig. 3). Accordingly, Turbo RFP did not show obvious toxicity in HeLa cells in three days (Fig. 5), and Bcl-xL had no effect on proliferation of cells expressing Turbo RFP (Supplementary Fig. 1D).

We also compared the average fluorescence intensity for cells transfected with DsRed, DsRed-Express2, Turbo RFP or GFP at 48 and 60 h, and the results showed that cells transfected with Turbo RFP or GFP shown much higher average fluorescence intensity than those of DsRed and DsRed-Express2 (Supplementary Fig. 2). The protein expression level can be estimated from dividing the average fluorescence intensity by the relative brightness of each fluorescent protein. As shown in Supplementary Fig. 3 [19–21], the expression levels of DsRed-Express2, Turbo RFP and GFP are comparable, and are about 10 times higher than that of DsRed. Considering that DsRed has much longer maturation time, even if only 10% of the expressed DsRed is matured, its expression level is just comparable to the other fluorescent proteins. Therefore, the difference in cytotoxicity is not related to the expression level of fluorescent protein.

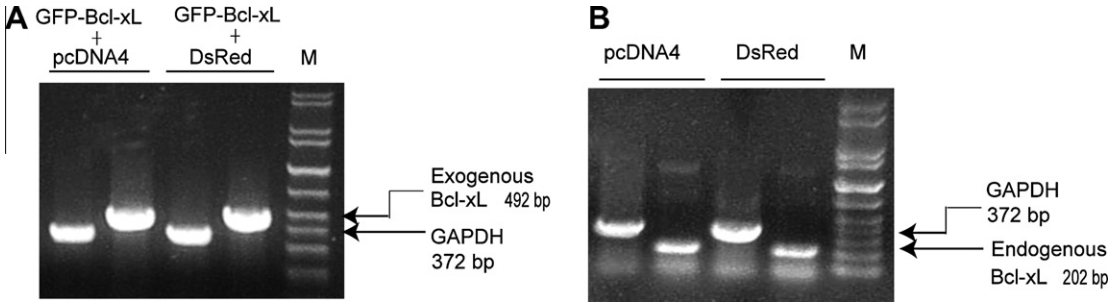

Fig. 4. DsRed did not inhibit the transcription of Bcl-xL in Hela cells. (A) RT-PCR assay of the transcription of exogenous Bcl-xL mRNA extracted from HeLa cells co-transfected with plasmids expressing GFP-Bcl-xL and DsRed or plasmids expressing GFP-Bcl-xL alone. (B) RT-PCR assay of the transcription of endogenous Bcl-xL mRNA extracted from HeLa cells transfected with plasmids expressing DsRed. Both exogenous and endogenous Bcl-xL mRNA levels were not affected by the overexpression of DsRed. GAPDH was used as an internal control. M: DNA ladder.

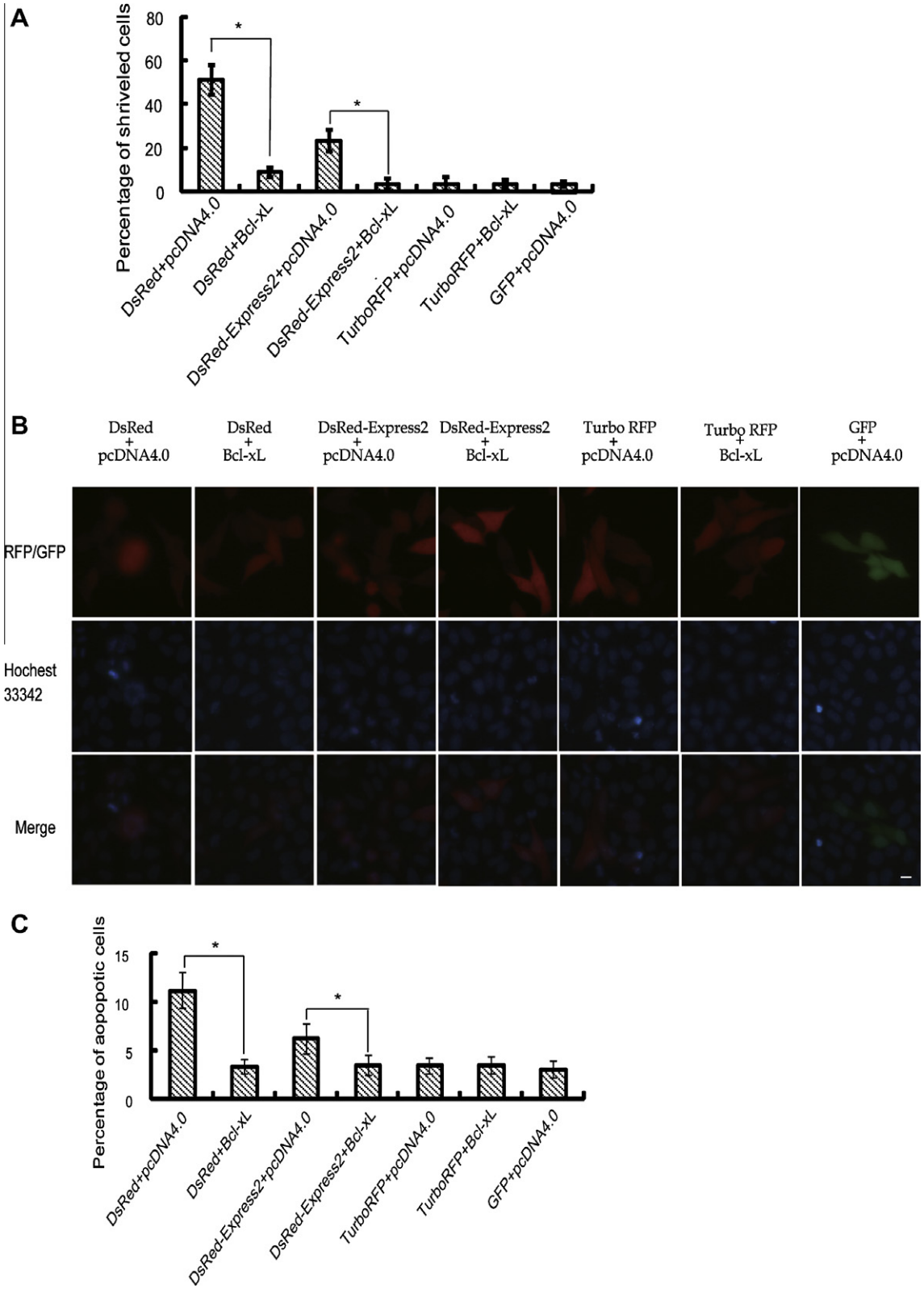

Fig. 5. Over-expression of Bcl-xL inhibits DsRed-mediated cytotoxicity. (A) The percentage of shriveled cells for HeLa cells co-transfected with plasmids expressing both GFP-Bcl-xL and DsRed/DsRed-Express2 or expressing DsRed/DsRed-Express2 alone, and cells expressing GFP alone were used as control. Over-expression of Bcl-xL inhibits DsRed and DsRed-Express2 elicited shriveled cells. (B) Representative fluorescence images of HeLa cells co-transfected with plasmids expressing both GFP-Bcl-xL and DsRed/DsRed-Express2 or DsRed/DsRed-Express2 alone, and cells expressing GFP alone were used as control. Hoechest 33342 was used for staining apoptotic cells. The bar represents 20 m. (C) The percentage of apoptotic cells for HeLa cells co-transfected with plasmids expressing both GFP-Bcl-xL and DsRed/DsRed-Express2 or expressing DsRed/DsRed-Express2 alone, and cells expressing GFP alone were used as control. $^{*}P < 0.01$.

4. Conclusion

In summary, we have demonstrated that DsRed and DsRed-Express2 can inhibit the expression of anti-apoptotic protein Bcl-xL, which results in cytotoxicity in Hela cells. Meanwhile, the over-expression of Bcl-xL inhibits DsRed-mediated cytotoxicity. Our results reveal a possible mechanism of DsRed cytotoxicity, further investigating the detail mechanism for DsRed and DsRed-Express2 on inhibition of Bcl-xL translation may help to alleviate the cytotoxic problem of DsRed and its variants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.02.013.

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